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1. Conner et al. (1983) PNAS 80: 278-282.

2. Rollini et al. PNAS (1985 Nov) 82(12): 7197-7201.

3.Gorski et al. IMMUNOGENTICS (1987) 25(6):379-402.

4.. de Preval et al. IMMUNOGEENTICS (1987) 26(4-5): 249-257.

5. Irle et al. J. EXPERIMENTAL MEDICINE (1988 Mar 1) 167(3): 853-872.

6. Andersson et al. IMMUNOGENETICS (1988) 28 (1): 1-5.

Thanks,
Lisa Arthur
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FUNCTIONAL POLYMORPHISM OF EACH OF THE TWO HLA-DR β CHAIN LOCI DEMONSTRATED WITH ANTIGEN-SPECIFIC DR3- AND DRw52-RESTRICTED T CELL CLONES

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The human class II genes encode heterodimeric glycoproteins that belong to at least three major HLA-class II families: the HLA-DR, -DQ, and -DP molecules. They play a central role in antigen presentation and in the control of immune responsiveness. Immune reactions are initiated after antigen presentation to the TCR by accessory cells (APC) in the context of class II molecules (1, 2).

Progress in the molecular genetics of HLA class II genes has clarified the genetic organization of the HLA-D region (3). The molecular basis for the allelic polymorphism at the individual class II loci has now been analyzed directly at the DNA sequence level and by oligonucleotide hybridization (4, 5). Two expressed DR β loci have been identified and linked in the HLA-DRw52 supertypic family (6). Locus DR βI encodes the well-known DR specificities DR3, w5, and w6, while locus DR βIII encodes the supertypic specificity HLA-DRw52 (7). The latter has recently been shown by DNA sequence analysis to include at least three alleles, called 52a, 52b (4), and 52c (Gorski, J., et al., manuscript in preparation), which can be identified by oligonucleotide typing (5, 8, 9). HLA-DR protein heterogeneity has also been observed by two-dimensional (2-D)[1] gel electrophoresis (10, 11).

The identification of this molecular and genetic HLA-DR β polymorphism in DRw52 haplotypes has prompted us to investigate in detail its functional aspect. To this end, T cell clones were isolated after in vitro tetanus toxoid (TT) restimulation of PBMC from immune donors. This study focuses on the correlation between the functional and the structural polymorphism within DRw52

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[1] *Abbreviations used in this paper:* CM, culture medium; 2-D, two-dim nsional; GAMIg, goat anti-mouse Ig; HTC, homozygous typing cell; TT, tetanus toxoid.

haplotypes, by comparing the pattern of reactivity of selected TT-specific, HLA-DR3- and DRw52-restricted T cell clones with the pattern of allele-specific hybridization with oligonucleotides in panel and family studies. The specificity of the antigen specific clones was also compared with that of the LB-Q1-alloreactive T cell line RT279 that was previously shown to recognize a subset of cells within DRw52 (12). The data presented show that the majority of the T cell clones isolated are restricted by epitope(s) of the DR βI locus (DR3), but that other clones are clearly restricted by DR βIII (DRw52) locus products. These clones can distinguish between the allelic products of locus DR βIII. The correlation between this functional polymorphism and the molecular basis for allelic diversity of the two DR β chain gene products is discussed.

Materials and Methods

Generation of T Cell Clones. Cultures were carried out at 37°C in an H_2O-saturated, 5% CO_2 atmosphere. RPMI 1640 (Gibco AG, Basel, Switzerland) supplemented with 15% human AB^+ serum from male untransfused volunteer donors and antibiotics was used as culture medium (CM). PBMC were obtained from volunteer donors by venipuncture aspiration of blood on heparin (10 IU/ml Liquemin Roche, lot number B0155 S.A.; F. Hoffmann-La Roche & Co AG, Basel, Switzerland) density centrifugation over Ficoll-Hypaque (No. 26239; Pharmacia Fine Chemical AB, Uppsala, Sweden), two washes in PBS, and suspension in CM. PBMC from selected donors were restimulated in 10 ml of CM and placed in 30-ml culture flasks (Corning 25100; D. V. Kobrin SA, Chambésy, Switzerland) as follows: 10^7 responders were cocultured with 10^7 autologous cells that have been previously incubated during 4 h with TT (Number FV.2; Institut Mérieux, Lyon, France), at a dilution of 1:2,500 (unless otherwise indicated), washed three times in CM, followed by irradiation (35 cGy). After 4 d of culture, the cells were harvested and T cell blasts were isolated by density gradient flotation using a Percoll gradient (Number 26239; Pharmacia Fine Chemical AB). Cells layering at the interphase between 50 and 55% (consisting mostly in blasts) were harvested, washed three times in CM, and used for cloning in limiting dilution culture. Autologous irradiated and TT-pulsed PBMC (10^4/well) were dispensed together with 0.3 blasts/well into 72-well microtiter culture grade Terasaki microculture trays (model F 3034 Microtest; Falcon Labware, Oxnard, CA) in a total volume of 20 μl of CM supplemented with 15% of an IL-2-containing culture supernatant (Lymphocult T, number 115074; Biotest Serum Institut, Frankfurt, Federal Republic of Germany). After 7 d the wells were inspected microscopically for growth, and confluent cultures were transferred into flat-bottomed, 96-well, 200-μl microculture trays (Falcon Labware 3070) containing Lymphocult-supplemented CM. Growing cultures were then tested for TT specificity with the autologous APC, incubated either with diphtheria toxoid, (1:5,000 dilution), candida antigen, (1:5,000 dilution), Proteus antigen, (1:100 dilution), trichophyton antigen, (1:5,000 dilution) (Institut Mérieux) tuberculin (40 U/ml; Statens Seruminstitute, Copenhagen, Denmark), or TT as described above.

HLA Typing. PBMC from volunteer blood donors were HLA typed using the standard National Institutes of Health (Bethesda, MD) complement-mediated microcytotoxicity assay (13).

Cryopreservation. PBMC and T cell clones were stored in the vapor phase of liquid nitrogen in 1-ml vials containing $5–10 \times 10^6$ cells suspended in CM supplemented with 10% DMSO (E. Merck, Darmstadt, Federal Republic of Germany).

Homozygous Typing Cells (HTC). PBMC from the HLA homozygous donors AVL, QBL, CAA, HHK, HAR were kindly provided by J. J. van Rood, Department of Immunohematology, University Medical Center, Leiden, The Netherlands, and by G. B. Ferrara, Institut Nazionale per la Ricerca sul Cancro, Genova, Italy).

Monoclonal Antibodies (mAb). We gratefully acknowledge the gift of the following mAbs for these studies. Their specificity and origin is indicated in brackets: Tu 22 (DQ)

and Tu 35 (DR; from A. Ziegler, reference 14), B7/21 (DP; from N. Reinsmoen, reference 15), D1/12 (DR; S. Carrel, 16), GSP4.1 (DR; Genetic Systems Corp., Seattle, WA, 17), 7.3.19.1 (DRw52-like; H. Bruning, 18), 16.23 (DR3 and some DRw6; J. J hnson, 19), NDS 10 (some DRw52; S. V. Fuggle, manuscript submitted for publication). These antibodies were provided in form of ascites and tested after dialysis against PBS and centrifugation. The dilutions of ascites that were tested are indicated in the figures of the result section. mAb.OKT-2, 3, -4, and -8 were purchased from Ortho Diagnostics Systems Inc., Westwood, MA.

Immunofluorescence. All manipulations were performed at 4°C. 10^5 cells were suspended in PBS containing 5% BSA and stained in two steps for indirect immunofluorescence at 4°C according to standard procedures. Cells were stained first with 5% goat serum–PBS and mAb, washed three times, then washed three times with goat anti–mouse Ig (GAMIg) conjugated to FITC (Coulter Clone; Coulter Immunology, Instrumenten Gesellschaft AG, Zürich, Switzerland) and suspended in 5 ml PBS for cytofluorometric analysis (EPICS-V; Coulter Electronics, Hialeah, FL). Control samples stained only with GAMIg-FITC were analyzed in parallel. At least 10^5 positive events were analyzed. The analyses were kindly performed by Dr. D. Wohlwend, Centre de Cytofluorométrie, CMU, Geneva, Switzerland.

T Cell Proliferation Assay. Freshly thawed T cell clones (2×10^4) were mixed with TT-preincubated washed and irradiated PBMC (5×10^4) and dispensed in 200 μl of CM into triplicate wells of a 96-well, round-bottomed microculture plate (Linbro No. 76-018-04; Flow Laboratories AG, Zug, Switzerland). After 48 h of coculture, 1 μCi of [^{3}H]TdR (TP 120; Amersham International Ltd., Amersham, UK) was added into each well and cultures were harvested on glass filters 8–12 h later. [^{3}H]TdR incorporation was determined by liquid scintillation spectroscopy. Results are expressed in counts per minute, unless otherwise indicated.

T Cell Functional Blocking Studies. Antigen-pulsed and irradiated stimulator cells were incubated with mAbs or CM for 1 h and washed three times, unless indicated otherwise. Responder clones (2×10^4) were added to 5×10^4 stimulators for a T cell proliferation assay (see above). The percent relative response of the responder clones to TT-APC in the presence of mAbs was calculated as follows: Percent relative response = 100 × [(cpm test + mAb)/(cpm CM control)]. Where *cpm test + mAB* is [^{3}H]TdR incorporation of mAb-exposed cultures in the presence of antigen, less [^{3}H]TdR incorporation of mAb-exposed cultures without antigen, and where *cpm CM control* is [^{3}H]TdR incorporation of CM-treated cultures with antigen, less [^{3}H]TdR incorporation of CM-treated cultures without antigen.

Stimulation with INF-γ. PBMC were cultured for 20 h before antigen exposure in the presence of 250 U/ml of rINF-γ (generous gift of Biogen SA, Geneva, Switzerland).

Oligonucleotide Typing. DNA was prepared as described previously by M. Gros-Bellard et al. (20) from the B cell lines AVL and QBL and from granulocytes or PHA blasts or LCL derived from volunteer blood donors who were HLA-typed in our laboratory. DNA was digested with Eco RI (Boehringer AG, Mannheim, Federal Republic of Germany), electrophoresed in 0.7% agarose gels in Tris-borate/EDTA buffer (pH 8.3) and transferred to Gene Screen Plus (New England Nuclear, Boston, MA) membranes in 0.4 M NaOH, 0.6 M NaCl. Oligonucleotide 52a probe is complementary to the sequence GGAGCTGCGTAAGTCTGAG of the HLA-DRw52a allele at positions 11–29 of the HLA DRβIII locus; oligonucleotide 52b is complementary to the sequence GTTCCTGGAGAGACACTTCC of the DRw52b allele at positions 62–81; and oligonucleotide 52c is complementary to the sequence GTTCCTGGAGAGATACTTCC of the DRw52c allele at positions 62–81 of the HLA DRβIII locus (Gorski, J., et al., manuscript submitted for publication; 4, 21). Oligonucleotides were end labeled with γ-[^{32}P]ATP (sp act = 5,000 Ci/mmol; Amersham International; 1Ci = 37 GBq) by T4 polynucleotide kinase (Pharmacia Fine Chemical AB) to a sp act of 5×10^8 cpm/μg. Prehybridization and hybridization with the labeled oligonucleotides (1.5×10^6 cpm/ml) were performed as described (5) except that dextran sulfate was omitted and only 5% NaDodSo4 (wt/vol) was used in both prehybridization and hybridizati n soluti ns. The membranes were washed for 1 h at 50°C (oligonucleotide 52b: 53°C) in 3× NaCl/Cit

(1× NaCl/Cit = 150 mM NaCl, 15 mM trisodium citrate, pH 7.0), 10× Denhardt's solution, 5% (wt/vol) NaDodSo4, 25 mM sodium phosphate (pH 7.0), and then for 1 h at 50°C (oligonucleotide 52b: 53°C) in 1× NaCl/Cit, 1% NaDodSo4 (9). Autoradiography was done for 5–15 d on preflashed X-AR5 Kodak films, using CAWO SE4 intensifying screens.

Results

Generation of T Cell Clones Specific for TT and Restricted by DR3-associated Epitopes. PBMC from two donors immune to TT (d.1417 and d.2778) were selected for these studies. These donors differed for the DR3-associated haplotypes (d.1417: HLA-A1,-;B8,21;DR3,-;DQw2,-/d.2778: HLA-A2,3;B5,12; DR3,6;DQw1,2). Their PBMC were restimulated in vitro with TT and cloned exactly as described (Materials and Methods). After cloning of d.1417 PBMC, 44 TT-specific clones (50% cloning efficiency) were obtained, and 11 TT-specific clones (cloning efficiency 19%) were derived from PBMC of d.2278. Of these 55 clones, 17 from d.1417, and 7 from d.2278 could be expanded sufficiently and screened for TT-specific proliferation against a panel of irradiated allogeneic PBMC from unrelated donors. This panel of PBMC was designed to allow selection of clones restricted by DR but not DQ determinants. We therefore included three DQw2 cells associated with non-DR3 haplotypes and two DQw1$^+$ cells, since d.2778 expressed DQw1, and both d.2778 and d.1417 the DQw2 antigens. Among the clones derived from d.1417 PBMC, four (clones 13, 16, 49, and 90) had maintained their reactivity to the autologous TT-pulsed cell. Among the clones from d.2778, three (clones 5, 9, and 10) were still reactive, and specific for TT; these clones were not stimulated by any of five other antigens (diphtheria toxoid, candida antigen, proteus antigen, trichophyton antigen, and tuberculin; see Material and Methods) tested using the autologous APC. All these clones were CD3$^+$,CD4$^+$,CD8$^-$. The results obtained with these seven informative clones are shown in Table I. None was restricted by DQw1-, and DQw2-associated epitopes. Clone 10 proliferated only to one DR3 TT-APC. This indicated restriction by a determinant distinct of DR3, or DQw, since none of the other TT-DQw2$^+$ DR3 or DR7 APC tested in this panel stimulated clone 10. More extended panel studies were therefore performed to define the restricting determinant of this clone.

The DR3-associated Restriction Polymorphism Defines Three Clusters within DRw52 TT-APC. The detailed specificities of these clones were explored against a panel of unrelated irradiated APC that covered a large spectrum of HLA-DR specificities. They were previously pulsed with TT or PBS. Clones 5, 9, 13, 90, and 49 reacted similarly and specifically to TT-DR3-APC. Representative results obtained with clone 49 are shown on Table II. This contrasted with the results obtained with clone 16 and clone 10. Clone 16 reacted specifically only to some TT-DR3-APC, and some TT-DRw6-APC. Interestingly, among four DR3 HTC (AVL, HAR, CAA, QBL) tested, QBL failed to present TT to clone 16. Clone 10 proliferated specifically to approximately half of the TT-DR3-APC, all TT-DR5-APC, and some TT-DRw6-APC. These TT-APC did not stimulate clone 16, except for three of the TT-DR5-APC that also expressed DR3, DRw52 encoded on the second haplotype (d.2757, d.521, d.891). Interestingly, clone 10

TABLE I

Proliferative Responses of Tetanus Toxoid (TT)-specific Selected Clones: Preliminary Screening for DQw2 Nonreactive Clones

Stimulator cells				[^{3}H]TdR incorporation*						
Donor number	HLA typing		TT	Clones from donor 1417				Clones from donor 2778		
	DR	DQw	+/−	13	16	49	90	5	9	10
				cpm × 10^{-3}				*cpm* × 10^{-3}		
895	7,9	2,3	+	0.4	0.6	0.2	0.3	0.3	0.4	0.9
			−	0.2	0.2	0.3	0.4	0.3	0.3	0.9
1213	6,7	1,2	+	0.2	0.3	0.1	0.4	0.1	0.2	1.3
			−	0.4	0.1	0.2	0.3	0.1	0.2	1.3
2644	4,7	2,3	+	0.6	0.3	0.5	0.3	0.1	0.1	0.7
			−	0.3	0.1	0.2	0.3	0.1	0.1	0.4
766	1,3	1,2	+	**1.5**	**2.1**	**6.3**	**2.6**	**18.6**	**13.0**	1.1
			−	0.4	0.3	0.2	0.1	0.2	0.2	0.7
22	3,10	1,2	+	**1.8**	**6.3**	**10.6**	**11.8**	**35.3**	**18.2**	0.7
			−	0.3	0.2	1.0	0.2	0.6	0.3	0.4
1675	3,1	1,2	+	**4.0**	**12.1**	**12.6**	**8.1**	NT	NT	NT
			−	0.4	0.4	0.6	0.1	NT	NT	NT
1417‡	3,−	2,−	+	**2.3**	**3.3**	**7.4**	**8.1**	NT	NT	NT
			−	0.4	0.4	0.4	0.2	NT	NT	NT
2190	3,4	2,3	+	NT§	NT	NT	NT	**46.2**	**84.5**	2.5
			−	NT	NT	NT	NT	0.5	0.3	1.1
2778‡	3,6	1,2	+	NT	NT	NT	NT	**17.0**	**8.1**	**21.3**
			−	NT	NT	NT	NT	0.1	0.1	0.7

* Results are expressed as means of triplicate cultures; standard deviation of the mean was always <20%. Results in boldface indicate positive data.

‡ These were used for cloning, resulting in the cell lines shown in the table.

§ NT, not tested.

was stimulated by TT presented by the HTC QBL, and failed to react with the other DR3 HTCs. Thus, the reactivity of clones 10 and 16 reflects recognition of mutually exclusive epitopes on TT-DRw52-APC.

The supratypic HLA-DRw52 specificity is almost perfectly associated with HLA-DR3, -5, -w6, and -w8 by serology. Clone 16 was not stimulated at all and clone 10 only very weakly by TT-DRw8 APC. This result indicates a significant functional difference between HLA-DRw52 associated with HLA-DRw8 and with HLA-DR3, -5, and -w6 haplotypes respectively. One TT-APC that expressed only one DRw52 allele (d.3135: HLA-DR2,w6;DRw52,-;DQw1,-) stimulated both clones 10 and 16. This cell (*a*) failed to stimulate the alloreactive LB-Q1-specific T cell line RT297, that otherwise overlapped completely with the pattern of reactivity of clone 10 (Table II), and (*b*) differed from the other DRw52 cells in the oligonucleotide typing experiments (see below and Fig. 4). This result suggests the existence of a third restriction epitope of DRw52 in some DRw6 haplotypes, in agreement with the identification of a third allele of DR βIII (Gorski, J., et al., manuscript in preparation).

Moderate alloreactivity of clone 10 to 3 DR5 cells (among a total of 21 tested DR5 APC) was n ticed (Table II). These three cells were DRw11. This alloreac-

TABLE II

Distinct HLA-DRw-52 Epitopes Are Associated with the Restriction for TT-reactive T Cell Clones

Donor	Stimulator cells: HLA typing A	B	DR	DQw	TT +/−	[^{3}H]TdR incorporation* (cpm × 10^{-3}): Clone number tested 49‡	16‡	10§	Alloreactive line RT 279§
AVL	1,1	8,8	3,3	2,2	+	15.3	1.9	0.7	—
					−	0.1	0.1	0.7	0.2
HAR	1,1	8,8	3,3	2,2	+	15.6	2.0	1.2	—
					−	0.1	0.1	1.2	NT¶
CAA	1,1	8,8	3,3	2,2	+	33.5	3.1	0.7	—
					−	0.1	0.2	0.7	NT
1417‡	1,–	8,21	3,–	2,–	+	31.8	3.3	0.5	—
					−	0.3	0.2	0.5	0.2
22	3,9	12,27	3,10	1,2	+	52.5	6.0	0.5	—
					−	0.2	0.2	0.3	0.2
1678	19,–	12,21	3,7	2,–	+	27.6	6.7	1.1	—
					−	0.3	0.2	0.5	0.2
2037	1,2	15,35	3,–	2,–	+	33.7	6.1	0.2	—
					−	0.1	0.2	0.1	0.2
31591	1,–	8,–	3,7	2,–	+	24.8	11.3	0.6	—
					−	0.1	0.1	0.6	NT
QBL	26,26	18,18	3,3	2,2	+	17.5	0.2	28.3	—
					−	0.4	0.2	0.6	19.9
1874	1,2	17,18	3,7	2,–	+	21.4	0.2	35.6	—
					−	0.2	0.2	3.0	23.1
2778§	2,3	5,12	3,6	1,2	+	20.9	0.3	45.4	—
					−	0.2	0.2	0.4	43.2
2757	1,3	7,8	3,5	2,3	+	54.8	7.0	58.2	—
					−	0.3	0.3	0.3	42.3
521	1,28	5,8	3,5	2,3	+	23.0	3.1	70.8	—
					−	0.2	0.2	4.5	44.9
NM	1,24	8,57	3,5	2,3	+	28.0	3.2	40.4	—
					−	0.1	0.2	0.4	11.7
891	2,9	15,22	3,5	2,3	+	65.2	1.5	90.6	—
					−	0.4	0.3	0.5	29.9
497	11,–	14,35	5,1	1,3	+	0.2	0.2	5.2	—
					−	0.3	0.3	0.5	33.8
673	1,19	12,17	5,2	1,3	+	0.3	0.3	60.0	—
					−	0.3	0.2	17.5	48.3
1165	3,10	7,12	5,2	1,3	+	0.2	0.5	27.7	—
					−	0.2	0.2	5.2	34.5
1104	9,24	7,12	5,2	1,3	+	0.5	0.3	37.8	—
					−	0.2	0.3	1.0	36.3
KD	2,–	5,12	5,4	3,–	+	0.2	0.2	10.7	—
					−	0.1	0.2	0.3	NT
2657	9,19	12,21	5,7	2,3	+	0.1	0.2	26.1	—
					−	0.2	0.1	0.2	19.8
JVM	2,2	18,18	5,5	3,3	+	0.2	0.1	30.7	—
					−	0.1	0.1	0.1	1.3
ATH	25,25	18,18	5,5	3,3	+	0.3	0.1	39.6	—
					−	0.2	0.1	0.2	16.0

TABLE II—*Continued*

Donor	Stimulator cells: HLA typing A	B	DR	DQw	TT +/−	[^{3}H]TdR incorporation* (cpm × 10^{-3}): Clone number tested 49‡	16‡	10§	Alloreactive line RT 279¶
WAP	2,31	18,18	5,5	3,3	+	0.2	0.1	**25.3**	—
					−	0.1	0.1	0.2	**19.7**
HHK	3,3	7,7	6,6	1,1	+	0.2	**3.3**	0.5	—
					−	0.2	0.2	0.4	0.1
1213	11,19	12,44	6,7	1,2	+	0.2	**2.1**	0.5	—
					−	0.2	0.1	0.4	0.2
2221	1,2	7,8	2,6	1,-	+	0.2	0.2	**33.4**	—
					−	0.2	0.2	0.7	**43.2**
3135	3,32	7,8	2,6	1,-	+	1.0	**10.7**	**13.9**	—
					−	0.2	0.2	0.4	0.3
2134	1,2	7,40	2,8	1,3	+	0.7	0.2	3.0	—
					−	0.1	0.1	0.2	0.3
2995	2,9	5,35	2,8	1,3	+	0.5	0.3	1.4	—
					−	0.1	0.2	0.2	0.2
1157	10,19	5,27	1,9	1,3	+	0.2	0.1	0.2	—
					−	0.3	0.3	0.4	0.2
2913	3,31	15,37	1,10	1,-	+	0.4	0.1	0.3	—
					−	0.3	0.3	0.5	0.3
ES	3,10	12,35	1,10	1,-	+	0.3	0.3	0.2	—
					−	0.2	0.2	0.1	0.3
1484	2,-	12,18	2,4	1,3	+	0.2	0.2	0.3	—
					−	0.3	0.2	0.5	0.1
658	2,3	7,21	2,7	1,2	+	0.3	0.3	0.5	—
					−	0.2	0.5	0.4	0.2
863	2,-	13,15	4,7	2,3	+	0.3	0.7	0.3	—
					−	0.2	1.0	0.1	0.2
ET	1,2	8,40	4,7	2,3	+	0.1	0.2	0.3	—
					−	0.1	0.2	0.2	0.1
895	1,2	13,27	7,9	2,3	+	0.2	0.3	0.7	—
					−	0.2	0.2	0.5	0.2

Representative panel of PBMC representing a wide spectrum of DR haplotypes, which were tested as APC in the presence (+), or absence (−) of TT using clones 49, 16, and 10 as responders, as described in Materials and Methods. These clones were TT-specific when tested with the autologous APC and six different antigens inciuding TT (see first section of Results and Materials and Methods). Boldface numbers represent cells 1417 and 2778 used for cloning, resulting in cell lines tested, and represent positive results obtained in panel experiments.

* Data shown represent means of triplicate cultures; the standard deviation of the mean was always <20%.

‡ Clones 49 and 16 were obtained from donor 1417.

§ Clone 10 was obtained from donor 2778.

¶ RT279 is specific for the LB-Q1 allodeterminant associated with DRw52.

* NT, not tested.

tivity was most probably not due to DRw11, since among 16 DRw11 cells no other one stimulated clone 10 in the absence of TT. Furthermore, no alloreactivity was observed with the DRw11 HTC JVM, which seems to represent a distinct DR5 (22), and which presented TT to clone 10. Since no pattern of association between alloproliferation and any HLA antigen was noticed, cross-

TABLE III

Independent Segregation of the Restriction Determinants of Clone 16 and Clone 10 in a Family Study

Donor	Stimulator cells: HLA haplotypes		TT-specific proliferation* Clone 16	Clone 10
Father:	a: A10, B7, DR1, -,	DQw1	0.3	**21.9**
	b: A28, B17, DR3, DRw52,	DQw2		
Mother:	c: A9, B15, DR2, -,	DQw1, -	**4.9**	0.2
	d: A28, B27, DRw6, DRw52,			
Sibling 1	b/d		**2.1**	**12.1**
Sibling 2	b/d		**3.5**	**27.7**
Sibling 3	a/d		**1.9**	0.3

* [^{3}H]TdR incorporation (cpm × 10^{-3}); standard deviation of the mean was always <20%. Results in boldface indicate positive data.

reactivity between self + TT and an epitope that remains presently undefined seems the most likely explanation of this observation.

Family Studies Demonstrate the Allelic Nature of the DRw52-related Restriction Polymorphism. The panel studies showed distinct DR3, 5, and w6 haplotype-associated restriction determinants included within the supratypic specificity DRw52. Several DRw52 heterozygous APC stimulated both clones 10 and 16. We therefore determined the segregation of the restriction with TT-APC from families where DRw52 heterozygosity occurred as a consequence of parental transmission of HLA-DR3-, -DR5-, and -DRw6-associated haplotypes. A typical result of such an experiment is shown on Table III. In this family (family R), as well as in two others tested, DRw52 was transmitted on two distinct parental haplotypes to the children. Clone 16 and clone 10 response patterns clearly allow to segregate TT-APC with distinct DRw52-associated haplotypes in the family. This demonstrates the functional significance of the allelic diversity within DRw52.

The DRw52 Specificity of the T Cell Clones Is Not Affected by Variations in Antigen Concentrations and HLA-DR Expression. Effective antigen presentation by APCs is thought to depend on the amount of antigen used during their incubation before testing, and on the level of HLA–class II expression (23–25). These factors were investigated using APCs that stimulated clone 16 or clone 10 in previous experiments. First, we tested the response of the clones to APCs that were incubated with TT dilutions ranging from 1:500 to 1:10,000. Increasing antigen concentrations resulted in increased proliferation (Fig. 1). Secondly, the influence of increased HLA–class II expression on the effectiveness of antigen presentation was explored after overnight incubations of APCs with INF-γ. Increasing the level of DR expression augmented the magnitude of the specific T cell clone response (Fig. 2). In these experiments the specific pattern of reactivity of clones 10 and 16 was not modified.

Inhibitor Effect of mAbs Specific for HLA–Class II Epitopes on Antigen-induced Proliferation of Clones 49, 10, and 16. Blocking of restriction determinants by mAbs can abrogate the response of antigen-specific, restricted clones. To confirm

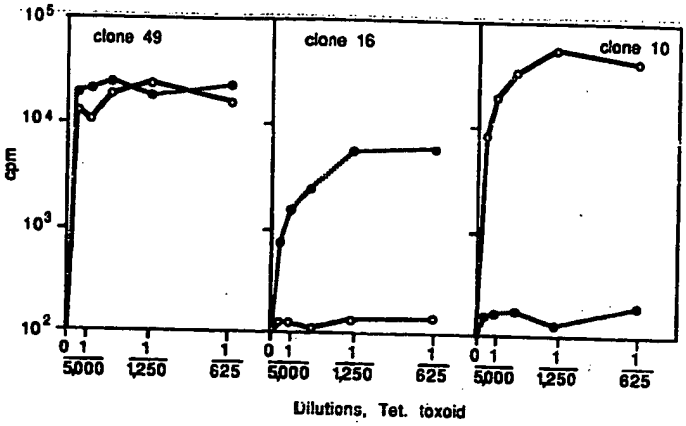

FIGURE 1. Proliferation of TT-specific T cell clones 49, 16, and 10 to TT-APC 1417 (autologous to clones 49 and 16, ●··●) and to TT-APC 2778 (autologous to clone 10, ○——○). For APC-HLA typing see Table II. Clone 49 reacts to both TT-APC (both DR3). Clone 16 and 10 react only to the autologous TT-APC, which express diverse DRw52 alleles. Antigen saturation does not induce reactivity of clone 10 and 16 to the opposite DRw52 alleles.

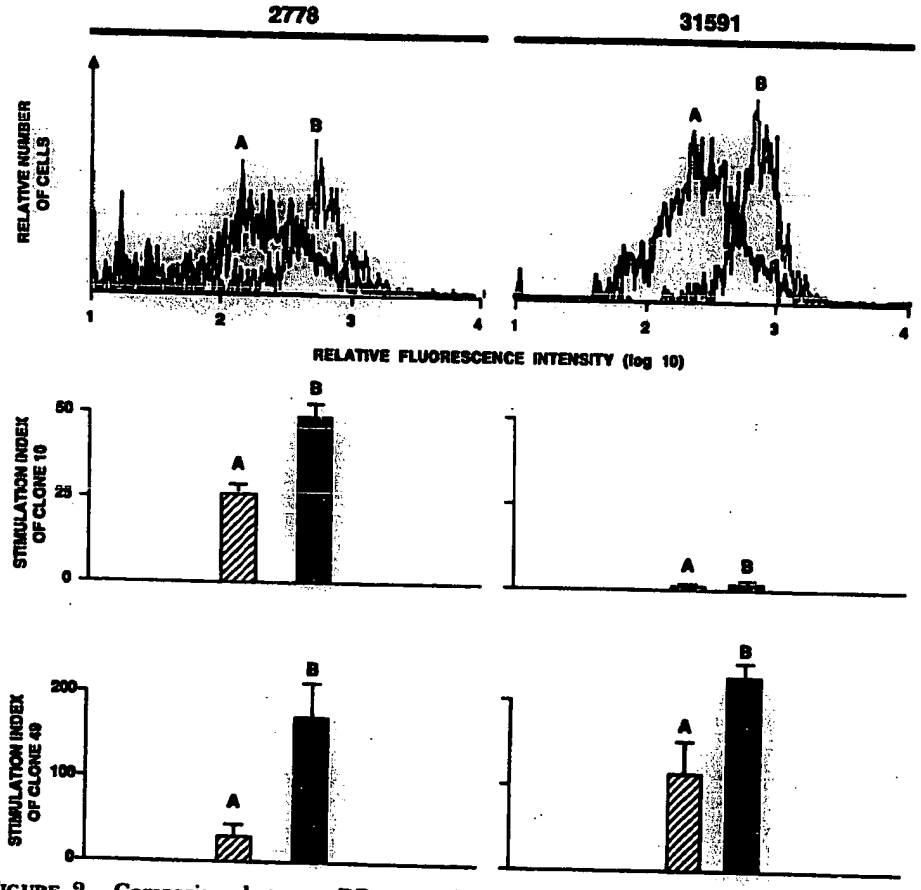

FIGURE 2. Comparison between DR expression evaluated by fluorescence flow cytometry analysis (FACS) and by antigen presentation after incubation of the APC in culture medium without (*A*, dark FACS lines) or with INF-γ (*B*, light FACS lines). Experiments with TT-APC from d.2778 (expressing the DRw52b allele), respectively d.31591 (expressing the DRw52a allele, oligonucleotide typing result not shown elsewhere) are shown. TT presentation is tested with clone 10, and clone 49, INF-γ increased DR expression by both APC (by ~0.5 log). The response of clone 10 to TT-APC 2778 increased significantly, and no response to TT-APC 31591 was seen, even after INF-γ. After this treatment, the response of clone 49 to both TT-APCs increased significantly.

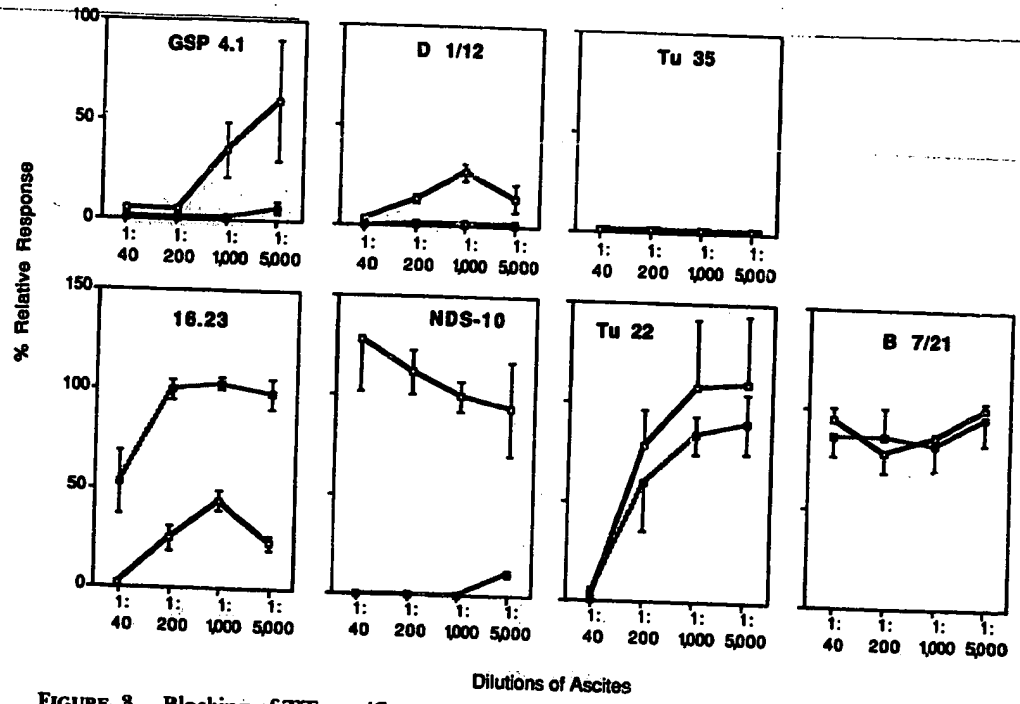

FIGURE 3. Blocking of TT-specific responses of clone 49 (■) and of clone 10 (□) to TT-APC (B-LCL DUCAF, HLA-DR3,3;DRw52,w52) by mAbs antibodies tested in increasing dilutions is shown. Results are shown in percent relative responses to TT-APC without mAb, and determined as indicated in Materials and Methods. Error bars represent the standard deviations of the triplicates. mAb GSP41 and D1/12 (DR framework) differentially block antigen presentation to both clones, in contrast to mAb Tu 35, which completely inhibits both clones at all concentrations tested. The mAb 16.23 (DR3 + some DRw6) inhibits clone 49 only, in contrast to the mAb NDS10 (DRw52-like) that inhibits only clone 10. mAb Tu 22 (DQ framework) dose not inhibit significantly at dilutions 1:200 and greater, and mAb B7/21 never blocked.

that the restriction determinants of clones 49, 10, and 16 relate to molecules of the DR family, and not to DQ or DP, T cell clones 49, 10, and 16 were tested for their specific proliferation to TT-APC incubated with mAbs specific for monomorphic determinants of the DR, DQ, and DP molecules. Some results of blocking experiments illustrating this point are shown in Fig. 3. All three clones were significantly blocked by anti-DR mAb GSP 4.1, D1/12, and Tu35. Differential blocking was observed between clone 49 and clones 10 and 16 (Fig. 3 shows clones 49 and 10 only, since inhibition of clones 10 and 16 were similar). Since DR3 and DRw52 epitopes are located on distinct DR molecules (7), the differential blocking is probably due to different framework antibody binding sites of these two molecules. Differential blocking by distinct mAbs of antigen-specific DR-restricted T cell clones was related by others (26) to recognition of functional epitopes on the DR molecules by some mAb only. The anti-DQ mAb Tu 22 inhibited the DR3- and the DRw52-restricted clones at very high concentrations (1:40). This inhibition was no longer seen when the antibody was diluted further. Since the anti-DP mAb B7/21 did not block these clones at similar concentrations, n nspecific inhibiti n seems unlikely. The best interpretation of

these findings is that weak binding of Tu 22 to a DR-associated epitope occurs at high antibody concentrations.

Additional evidence that the restriction determinants of the clones mapped to DR3, respectively to DRw52 alleles, was obtained in blocking experiments with mAb 16.23 (specific for DR3 and some DRw6), NDS10 (specific for some DRw52), and 7.3.19.1 (specific for a DRw52-like epitope). The mAb 16.23 blocked the DR3-restricted clone 49 but not the DRw52-restricted clone 10. In contrast, mAb NDS10 blocked clone 10, but not clone 49. The mAb 7.3.19.1 blocked all the clones (data not shown), suggesting binding to an epitope shared by both DR molecules. These results provide additional evidence for distinct functional epitopes of DR molecules containing the DR3 and the DRw52 specificities.

Correlation between the Reactivities of Clones 10 and 16 and the Allelic Series Defined at Locus DR βIII by Oligonucleotide Hybridization. The experiments described above provide evidence for functional epitopes that map to distinct alleles of DRw52. These are associated with the haplotypes of the HTCs AVL, QBL, and d.3135, respectively. DNA sequencing studies have shown that the DR3 HTC AVL and QBL are respectively 52a and 52b at their DR βIII locus, and have identified a third allele named 52c in some DRw6 haplotypes (Gorski, J., et al., manuscript in preparation).

Since the T cell clones described here also distinguish AVL and QBL, we have determined the segregation of 52a and b alleles as well as of the 52c allele by oligonucleotide typing in panel cells and families that had been studied with clones 49, 10, and 16. A selection of results obtained using the cells listed in the panel studies (represented in Table II) are shown on Fig. 4. These studies demonstrate a complete correlation between the DRw52a oligonucleotide hybridization pattern and the reactivity of clone 16. Similarly, there was a complete overlap in the hybridization of the oligonucleotide specific for 52b and the reactivity of clone 10. The d.3135 TT-APC that had stimulated both clones but not the alloreactive LB-Q1-specific T cell line RT279, was typed DRw52c using the DRw52c oligonucleotide probe. The epitope recognized by the LB-Q1-specific T cell line RT279 is absent from this allele.

The correlation between the functionally defined determinants restricting the TT-specific reactivity of clone 16 and of clone 10 with the 52a and the 52b alleles defined by oligonucleotide typing in the panel experiments was confirmed in family studies. The results obtained with one informative family (family R) are shown (Fig. 5). These results demonstrate the functional and structural correlation between the alleles of locus DR βIII.

Discussion

Within the class II region of human HLA haplotypes, the HLA-DR subregion contains a variable number of β chain genes differing in their polymorphism (3, 4, 8). The DR restriction of antigen-specific T cell responses is believed to be associated with the highly polymorphic β gene chain, that most likely contains the epitope(s) recognized by the alloantigen-specific DR sera. This poses the question of the functi nal role f the less polym rphic βIII locus expressed in most haplotypes. We have shown recently with the use of transfected L cells that

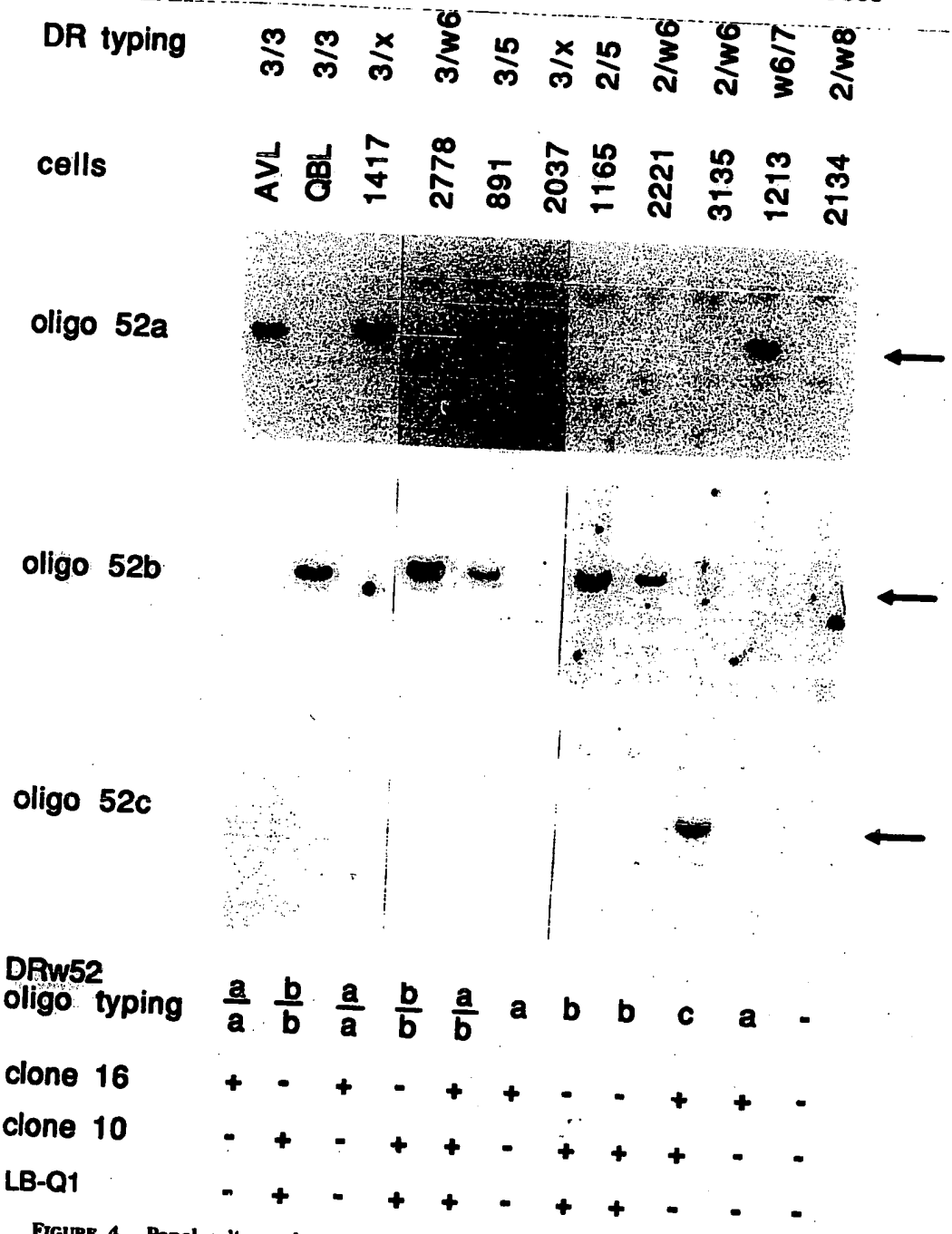

FIGURE 4. Panel cells used to characterize clones 49, 10, and 16 were analyzed using oligonucleotide DNA typing for DRw52a, 52b, and 52c alleles. The oligonucleotide sequences and their uses are described in Materials and Methods. Serological DR typing and cell identification numbers are indicated above each lane. Arrows point to the Eco RI 3.3-kb fragment corresponding to the first domain exon. The oligonucleotide typing results (52a, b, or c) are indicated under each lane, together with the reactivity (+/−) of each of the cells with clones 10 and 16, as described in Table II. Expression of the LB-Q1 determinant, recognized by the T cell lin RT279, is also indicated.

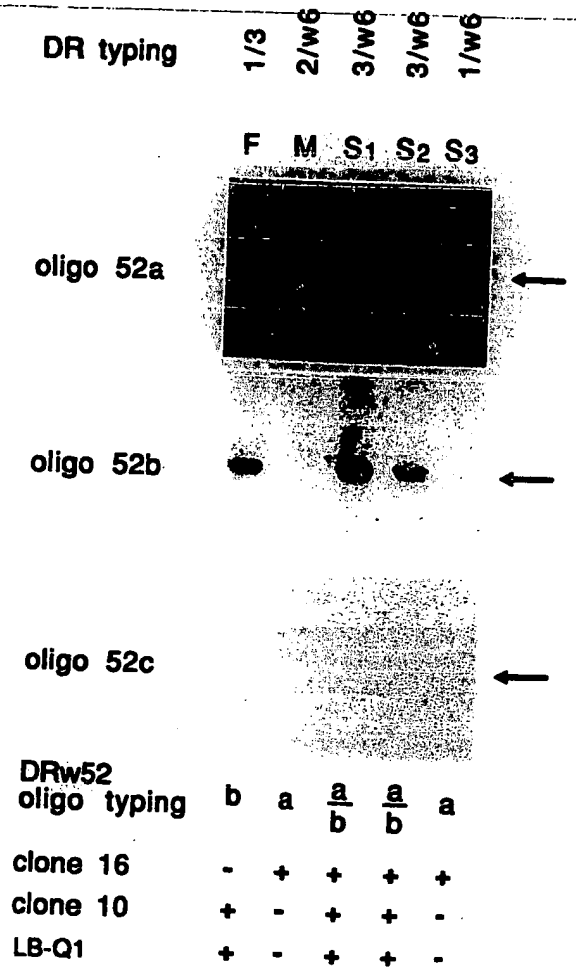

FIGURE 5. Oligonucleotide DNA typing analysis of the family R for DRw52a, b, and c alleles performed as described in Materials and Methods. Each lane contains Eco RI–digested genomic DNA from family members whose DR typing is shown. (F, father; M, mother; S1–S3, siblings). The typing results are indicated under each lane, and compared with the results obtained with clones 10 and 16. Expression of the LB-Q1 determinant, recognized by the T cell line RT279, is also indicated.

this DR βIII locus encodes the serologically defined supratypic DRw52 specificity (7) and that three alleles of βIII can be recognized by DNA sequencing (4, and Gorski, J., et al., manuscript in preparation). These three alleles can now be identified by oligonucleotide typing (5, 8, 9). Studies with alloreactive and antigen-specific T cell lines have also indicated the existence of polymorphism within DRw52 haplotypes (12, 27, 28, and Sheehy, M. J., personal communication). The relation between functional MHC restriction polymorphism, MHC polymorphism recognized by alloreactive T cells, and structural MHC polymorphism is presently unclear. We have therefore explored the functional relevance of each of the DR β chain loci with antigen-restricted T cell clones.

The correlation between these functional studies and oligonucleotide typing of the panel and family cells indicated that the restriction determinants for TT could be mapped to the polymorphic domains of the DR βI as well as the DR βIII gene products. This was shown by the direct correlation between the response pattern of clone 49 and DR3, between clone 16 and the 52a-specific oligonucleotide, between the response pattern of clone 10 and the 52b specific

probe, and between the response by both clones to d.3135 that typed 52c with the specific oligonucleotide. Since these clones are restricted by epitopes mapping t each of the DR β loci, the DR βIII gene product is therefore functional and contributes to antigen-specific T cell responses.

Previous indications of heterogeneity within DRw52 were obtained with alloreactive T cell clones (12, 27, 28, and Sheehy, M. J., personal communication), and with mAbs (11). However, most determinants that can be recognized by such reagents are not relevant for antigen presentation, which seems to be the primary function of MHC molecules. Indeed, restriction by class II molecules may only involve a single antigen binding site (1, 29). In this respect, the present studies differ fundamentally from these previous reports of DRw52-associated diversity, since they are based on antigen presentation, and therefore describe a functionally relevant polymorphism within DRw52. Our results also indicate a direct correlation between these newly recognized functional epitopes and the structural polymorphism of the DR βIII genes identified by DNA sequencing and by locus- and allele-specific oligonucleotide probes.

The supertypic DRw52 specificity comprises not only DR3, 5, and w6 but also DRw8. The lack of significant reactivity of our DRw52-restricted allele-specific clones to all TT-DRw8-APC tested, together with the lack of stimulation of the alloreactive LB-Q1-specific T cell line RT279 by DRw8 cells (12, and results shown in Table II), and the failure of several mAbs specific for DRw52-associated epitopes to recognize DRw8 cells (18, Fuggle, S. V., manuscript submitted for publication) suggest a structural particularity of DRw8. The failure of DRw52 locus-specific oligonucleotide probes to hybridize with DNA from two of these DRw8 cells analyzed suggests that the serologically defined DRw8-associated DRw52 specificity in not due to any one of the three alleles identified at locus DR βIII by these reagents. Since RFLP studies of DRw8 cells suggest the presence of only a single DR β chain gene (30), the best interpretation is that DRw8 individuals have only one DR βI locus whose product is recognized by DRw52 antisera through a DRw52 framework epitope.

The main restriction determinants for antigen presentation appear to relate to HLA-DR, rather than to HLA-DQw, or -DP gene products, although these latter can function as restriction determinants (31–33). Despite the considerable heterogeneity among the polypeptides in TT preparations, allowing for multiple antigen–class II associations, the functional DR specificities seem to occur primarily with the product of the DR βI gene. This is suggested by the more frequent finding of DR3-restricted T cell clones compared with DRw52-restricted T cell clones in several cloning experiments with cells from different donors. The possibility of immunodominance of DR βI–controlled restriction could reflect differences in the level of expression of the DR antigens rather than intrinsic functional differences, or a bias in the T cell repertoire, or receptor affinity. This view is supported by recent findings showing that the DR βI locus is expressed about three- to fivefold more than locus DR βIII at the mRNA level in peripheral blood lymphocytes and monocytes (34). Sequential immunoprecipitation studies have also suggested an excess of DR βI gene product (25). Finally, we show that INF-γ-treated APC that express more DR molecules have an increased efficacy in TT presentation t T cell clones, and this for both DR β

gene products. The influence of the level of MHC expression, including its abberant expression on T cell responsiveness was already illustrated by several experiments in animal and human systems (23, 24, 35). These observations point out that modifications in the regulation of HLA–class II gene expression might influence the pattern of T cell reactivity through an increased availability of functional class II epitopes. This could play a crucial role in the pathogenesis of autoimmunity (3).

The function of the DR βIII locus product in antigen presentation and in allelic restriction is of particular interest in view of the drastically different patterns of amino acid sequence diversity between the DR βI and the DR βIII gene products. This is illustrated in Fig. 6 with two examples. In the DR3, -5, and -w6 haplotypes, DR βI alleles differ almost exclusively at a small hypervariable segment around amino acids 67 and 74, while the alleles of locus DR βIII vary by dissimilarities that are scattered throughout the first domain (3, 4). Therefore, the finding of antigen-specific T cell clones restricted by either locus DR βI or DR βIII, and discriminating between the newly recognized DRw52 alleles at locus DR βIII, can help us to understand the structural nature of the DR determinants responsible for antigen presentation to T cells.

The basis for this isotypic and allelic distinction by antigen-specific T cells is the amino acid sequence of each of the relevant DR β chains (DR3 βI, DRw6 βI, DRw52 βIII a, b, and c). These are analyzed in Fig. 6. Despite the considerable sequence homology between the DR βI chain of DR3 and DRw6, no functional crossreactivity occurred between the restriction sites encoded by these two alleles. As discussed elsewhere (4), a gene conversion event has resulted in the introduction of two novel amino acids from a DR βIII 52a sequence into a DR βI DRw6 sequence, and this new DR β chain sequence determines the DR3 βI specificity. DR3-restricted T cell clones recognize only the product of the converted gene (DR3 βI), and not the DRw6 βI or βIII products. Furthermore, the DRw52a-restricted clone 16 does not react with the DR3 βI product (expressed on DR3/DRw52b cells), despite the presence within the DR3 βI polypeptide of elements of the 52a sequence (Fig. 6). The basis for T cell discrimination between DR3 and DRw6 is therefore not the individual amino acid sequence but rather the conformational change in the DR βI chain modified by conversion (4). This interpretation is also in agreement with studies correlating structure and function by site-directed mutagenesis. These experiments showed that conformational changes that modify H-2 specificities can arise at a site that is distant from the mutagenized region (36–38).

The structural basis for the functional discrimination between the newly recognized DRw52 alleles at locus DR βIII can also be deduced from the amino acid sequence comparisons. In contrast to the clustering of sequence differences between βI locus products in the hypervariable region defined between a.a. in position 67–74, differences between βIII alleles are scattered throughout the first domain and only one amino acid difference (R/Q) is located within the segment corresponding to the hypervariable region of βI (Fig. 6).

The structural basis for functional allelic determinants might thus be different for DR βI and DR βIII gene products. Recent experiments have suggested that the physical basis for restriction might result from qualitative differences in the

FIGURE 6. Nucleic acid sequence–deduced amino acid sequences of the NH_2-terminal domains of the DR βI gene chains of HTC AVL (DR3) and HTC HHK (DR6), and the DR βIII gene chains of the HTC AVL (DRw52 a) and QBL (DRw52 b). Boxes show regions of allelic sequence polymorphism. Notice that almost all sequence differences in the DR βI chain occur between amino acids 67 and 77, while they are widely scattered in the DR βIII chain (examples shown are 52a and 52b). Hatched boxes indicate the areas recognized by the oligonucleotides specific for DRw52 a, b, and c. The oligonucleotide sequences are indicated in Materials and Methods.

interaction between antigen and an antigen binding site on class II molecules (1, 29, 39, 40), each class II molecule presenting a limited number of antigenic peptides selectively (40). The basis for restriction seems therefore to be a consequence of complementarity between a segment of the class II molecule itself and the antigen, rather than direct class II recognition by the TCR (1, 41). Since restriction is linked to DR polymorphism, the antigen binding site of class II molecules is probably encoded by the polymorphic region. In this respect, it is of interest to observe that DR βIII–encoded class II molecules are functional, although they have a very distinct pattern of allelic structural diversity (sequence differences are scattered) when compared with sequence differences between DR βI products that are limited to a short hypervariable segment. This distinction in the structural basis of polymorphism between DR βI and DR βIII could result in a difference of their number of antigen binding sites, thereby increasing the chances of effective antigen–class II interaction.

Summary

HLA-DR3- and HLA-DRw52-associated functional polymorphism was investigated with selected tetanus toxoid (TT)-specific T cell clones. We have shown earlier that HLA-DR antigens are encoded by two distinct loci, DR βI and DR βIII. The alloantigenic determinant(s) defined by the serological HLA-DR3 specificity map to the former, while the supratypic HLA-DRw52 determinants map to DR βIII. Furthermore, we have recently recognized by DNA sequencing three alleles of HLA-DRw52 at locus DR βIII, referred to as 52 a, b, and c.

Our objective was to correlate the pattern of T cell restriction with the gene products of individual DR β chain loci and with the three newly described alleles of locus DR βIII. Among the selected T cell clones, 5 reacted exclusively when TT was presented by HLA-DR3$^+$ APCs (TT-DR3-APC). In contrast, two T cell clones were stimulated by TT-DRw52-APC. More specifically, these two T cell clones (Clones 10 and 16) were stimulated by different subsets of TT-DRw52-APC. Clone 16 responded to some DR3 and TT-DRw6-APC, while clone 10 was stimulated by other TT-DR3 and TT-DRw6, and all TT-DR5-APC. This same pattern of DRw52 restriction was found in panel, as well as in family studies. Because this suggested a correlation with the pattern of DRw52 polymorphism observed earlier by DNA sequencing and oligonucleotide hybridization, the APC used in these experiments were typed for the 52 a, b, and c alleles of locus DR βIII by allele-specific oligonucleotide probes. This distribution overlapped exactly with the stimulation pattern defined by the T cell clones. Clone 16 responded to TT-52a-APC, clone 10 to TT-52b-APC, and both clones to a TT-52c-APC. The response of the T cell clones was inhibited differentially by mAbs to DR. Raising TT concentration, or increasing HLA-class II expression with INF-γ both affected the magnitude of response of the TT-specific clones but did not modify their specificities.

These results demonstrate that a restriction specificity can be attributed to the DR βIII locus and illustrate the functional relevance of the polymorphism observed at this locus. This is of special interest in view f the striking difference in the pattern of structural diversity among alleles of DR βI and DR βIII.

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